A Preliminary Immunologic Study of Urinary Proteins:
The Questionable Value of Protein Clearances in Kidney Disease

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ABSTRACT

The clearances of seven different proteins were measured by a quantitative immunodiffusion technique in 15 patients with proteinuria. All urines were also studied by immunoelectrophoresis.

The renal histology was evaluated in each case, and no correlation was found between histologic changes and the urinary protein excretion. This observation was confirmed by both immunodiffusion and immunoelectrophoretic techniques. No specific urinary protein excretion pattern was found in six patients with systemic lupus erythematosus.

High-molecular-weight proteins were rarely found in urine, even when the glomerular basement membrane was definitely thickened. Low-molecular-weight proteins were often observed, but their clearances were variable. The results do not support the suggestion that protein clearances are valuable diagnostic and prognostic tools in renal diseases. They also do not support the view that glomerular filtration is the sole factor responsible for the final patterns of urinary proteins; tubular reabsorption is probably another important factor.

SOMMAIRE

Les "clearances" de sept protéines différentes furent mesurées chez 15 malades atteints de protéinurie à l'aide d'une méthode quantitative basée sur le principe de l'immunodiffusion en milieu gélifié. De plus, l'analyse immunoélectrophorétique de l'urine fut pratiquée dans chaque cas.

Chaque malade fut soumis à une biopsie rénale transcutanée; aucune corrélation n'a pu être établie entre les modifications histopathologiques rénales et l'excrétion urinaire des protéines. L'étude de six malades souffrant de lupus érythémateux disséminé n'a révélé aucune élimination protéinique d'un trait caractéristique de cette maladie.

Les protéines à poids moléculaire élevé furent rarement décelées dans l'urine, même lorsque le changement histologique prédominant était un épaississement de la membrane basale glomérulaire. Les protéines à faible poids moléculaire furent au contraire fréquemment observées, mais leurs "clearances" étaient des plus variables. La suggestion émise par d'autres auteurs que les "clearances" des protéines ont une grande valeur dans le diagnostic et le pronostic des maladies rénales ne semble pas confirmée par les résultats de la présente étude. En outre, les résultats actuels vont à l'encontre de l'hypothèse qui assigne à la filtration glomérulaire un rôle prédominant dans la formation définitive des traits caractéristiques des protéiniques urinaires; la réabsorption tubulaire des protéines est un autre facteur important.

STUDIES of proteins have been facilitated greatly by the recent introduction of immunologic methods, which have made identification and quantification of specific proteins relatively easy, even in the presence of a complex mixture such as human serum. Because of their high sensitivity and specificity, immunologic techniques are superior to the more classical methods such as electrophoresis, salting-out precipitation and even chromatography. The variety of proteins which can be studied is limited only by the number of available immune sera.

Immunologic methods have been used by Rowe and Soothill[1, 2] and by Blainey *et al.*[3] in studies of urinary proteins. Hardwicke and Soothill[4] investigated the possible relationship between renal histologic changes and glomerular permeability to proteins in patients with kidney diseases, and concluded that immunologic analysis of urinary proteins was of diagnostic value. In order to reassess this method we have undertaken a similar study; we also wished to determine whether or not certain diseases, and particularly systemic lupus erythematosus (SLE), were characterized by definite patterns of protein excretion.

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MATERIAL

Urinary proteins were studied in 15 patients, all of whom had clinical and/or laboratory evidence

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of kidney disease. A diagnosis of SLE was made in six of these patients.[5] In each case a percutaneous renal biopsy was performed.[6] Membranous glomerulonephritis was present in seven cases and proliferative glomerulonephritis in six. In two children with the nephrotic syndrome there were no glomerular abnormalities by light microscopy. In the patients with SLE there were both cellular proliferation and thickening of the glomerular basement membrane; basement membrane thickening predominated in three and cellular proliferation in the other three.

METHODS

Concentration of urinary proteins

Twenty-four-hour urine specimens were collected in refrigerated bottles with 1 g. of thymol as a preservative. They were kept frozen until the time of study, when they were thawed rapidly and filtered, and the protein concentration was determined by the biuret method on TCA-precipitates. Aliquots were then concentrated with an ultrafiltration apparatus using positive pressure and cellulose membranes with a pore size less than 5 mμ.* This device was used by Schröer and Imhof[7] to concentrate cerebrospinal fluid. By this method, excess salts are not present in the concentrates; after a brief period of dialysis against distilled water subsequent lyophilization of specimens is easily done. The lyophilized material was then solubilized in a minimal volume of water. With this technique, urines were concentrated between 10 and 200 times (mean: 60), with a final protein concentration varying between 3.8 and 66.2 g. per 100 ml. (mean: 19.6). Details of this method will be published elsewhere.[8]

Immunodiffusion technique: protein clearances

Protein clearances were estimated by the double diffusion method in agar gel as described by Soothill.[9] This technique permits a direct measurement of the urine/plasma (U/P) ratio of a protein without knowledge of the plasma concentration. Clearances can be derived from the U/P ratios when urine flow rates are known. However, to make results more comparable from one subject to another, clearances were expressed as a percentage of the respective albumin clearances. The study of IgA globulin (β_2A) (Fig. 1) illustrates some aspects of the immunodiffusion technique.

In the present study, U/P ratios of eight different proteins (molecular weights: 69,000 to 3,000,000[10]—Table I) were measured in all 15 patients. Commercial immune sera† were used throughout the study. Prior to analysis, the specificity and potency of each lot were carefully verified by both immunodiffusion and immunoelectrophoresis. Albumin and transferrin were measured in the original urines. Concentrated urine specimens were used for all other studies.

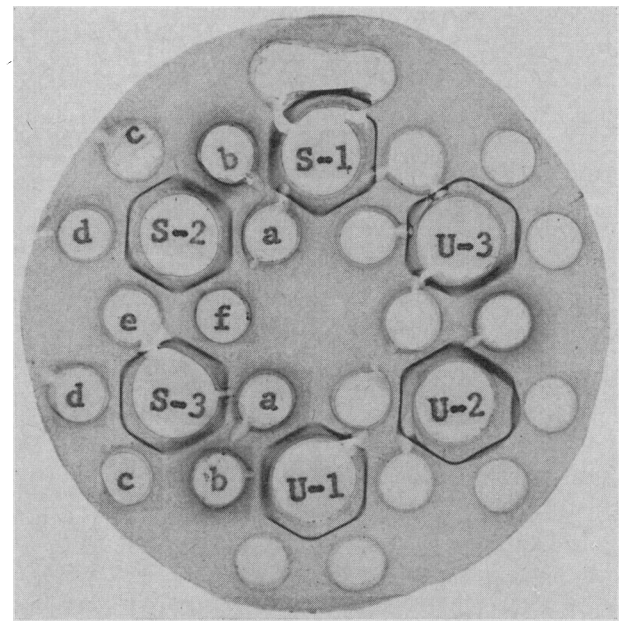

Fig. 1.—Immunodiffusion study of IgA globulin. The immune serum has been placed in the small peripheral wells in the following concentrations: (a) 100%, (b) 75%, (c) 50%, (d) 33%, (e) 25%, and (f) 20%. The patient's serum was deposited in the central reservoirs S-1, S-2 and S-3 in 1:4, 1:5 and 1:7 dilutions, and the concentrated urine (× 100) in reservoirs U-1, U-2, U-3 in 1:3, 1:4 and 1:5 dilutions. Because the S-2 hexagon matches that of U-1, it is concluded that S 1:5 = U 1:3. Since the U/P ratio of albumin was 1/8 using the original urine, the ratio $C_{IgA}/C_{albumin} \times 100 = 4.8\%$.

TABLE I.—MOLECULAR WEIGHTS OF PROTEINS STUDIED

1—Albumin	69,000
2—Transferrin	90,000
3—Beta-2A (γ_1A)	160,000
4—Gamma (7S, γ_2)	160,000
5—Alpha-2M	820,000
6—Beta-2M (19S, γ_1M)	1,000,000
7—Beta lipoprotein	2,500,000
8—Alpha-2 lipoprotein	3,400,000

Immunoelectrophoresis

Immunoelectrophoresis was performed according to a technique previously described[11] in a Shandon universal electrophoretic cell,* using barbital buffer (pH 8.6) and a current of 15 mamp. per glass plate.

RESULTS

Protein clearances

The results of the protein clearances are summarized in Table II. The clearances for particular proteins varied over a wide range of values even with the same histologic group. For example, $C_{transferrin}/C_{albumin}$ varied between 2% and 800% in proliferative glomerulonephritis and between

*Model MD 70 and Membrane Filters LSG 60, C. Schleicher & Schuell, Keene, New Hampshire, U.S.A.
†Behringwerke AG; Hyland.

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TABLE II.—PROTEIN CLEARANCES AS MEASURED BY THE IMMUNODIFFUSION TECHNIQUE

	Histologic types		
	Proliferative glomerulo-nephritis	*Membranous glomerulo-nephritis*	*Minimal changes*
	($C_{Prot.}/C_{Alb.}$ *in per cent*)		
Transferrin	2 25 50 100 530 800	18 20 25 54 100 234 300	160 312
Beta-2A	1 13 134 U.D.(3)	2 5 11 66 U.D.(3)	6 8
7S-Gamma	5 9 14 168 U.D.(2)	0.4 0.5 8 16 25 28 U.D.(1)	4 10
Alpha-2M	6.6 12 U.D.(4)	0.1 6.6 U.D.(5)	U.D. (2)
Beta-2M	2 7 U.D.(4)	0.4 6 U.D.(5)	0.4 1.3
Alpha-2 lipoprotein	100 240 U.D.(4)	22 U.D.(6)	U.D.(2)

U.D.: Immune precipitates were undetected in the number of urine specimens indicated in parentheses.

18% and 300% in membranous glomerulonephritis. As the molecular weight of transferrin is somewhat higher than that of albumin, it is perhaps surprising to observe that $C_{transferrin}$ was equal to or higher than $C_{albumin}$ in three of the six patients with proliferative glomerulonephritis and in the two patients with minimal histologic changes. The clearance of IgA (β_2A) and IgG (γ_2) globulin was higher than that of albumin in a patient with proliferative changes. In addition, the clearance of high-molecular-weight α_2-lipoprotein clearance was higher than $C_{albumin}$ in two cases of proliferative glomerulonephritis.

In general, however, the very high-molecular-weight proteins were rarely found in urine. Alpha$_2$-M, α_2-lipoprotein and IgM (β_2M) were absent, respectively, from 11, 12 and 9 of the 15 specimens. The results of the β-lipoprotein clearances were incomplete and are not tabulated. Immune precipitates could not be detected in many instances even when the most concentrated specimens were tested. These negative results do not exclude the possibility that these proteins were present at very low levels undetectable by the immunodiffusion method.

Relationship between protein clearances and histologic findings

From Table II it is evident that specific patterns of protein clearance did not correlate with the renal histologic findings. Protein clearances were variable in patients with proliferative glomerulonephritis and in those with membranous lesions. Also, the appearance of large-molecular-weight proteins in the urine was not restricted to any one histologic group. There was also no characteristic pattern in the patients with SLE.

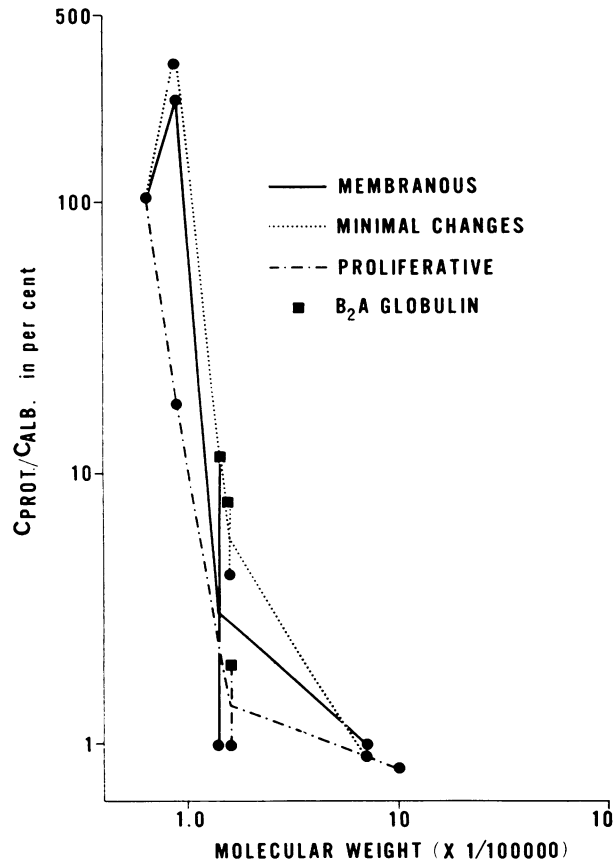

Fig. 2.—Selectivity slopes of proteinurias in three patients with different renal histologic changes. The similarity between these curves is to be noted.

Joachim *et al.*[12] have proposed that the relationship between relative protein clearances and molecular weight be expressed as regression lines in order to define the degree of selectivity of proteinuria. Expressed as percentages of $C_{transferrin}$, protein clearances were plotted as ordinates against respective molecular weights on a log-log scale. Linearity of the curves was estimated by the method of least squares and the degree of selectivity expressed as an angle derived from a horizontal tangent to the slope. Fig. 2 illustrates such "selectivity" slopes in three patients with different types of glomerular lesions. Although the histologic findings differed in all three patients, the three curves are remarkably similar. Furthermore, the validity of expressing results in terms of selectivity slopes is doubtful, when values such as those

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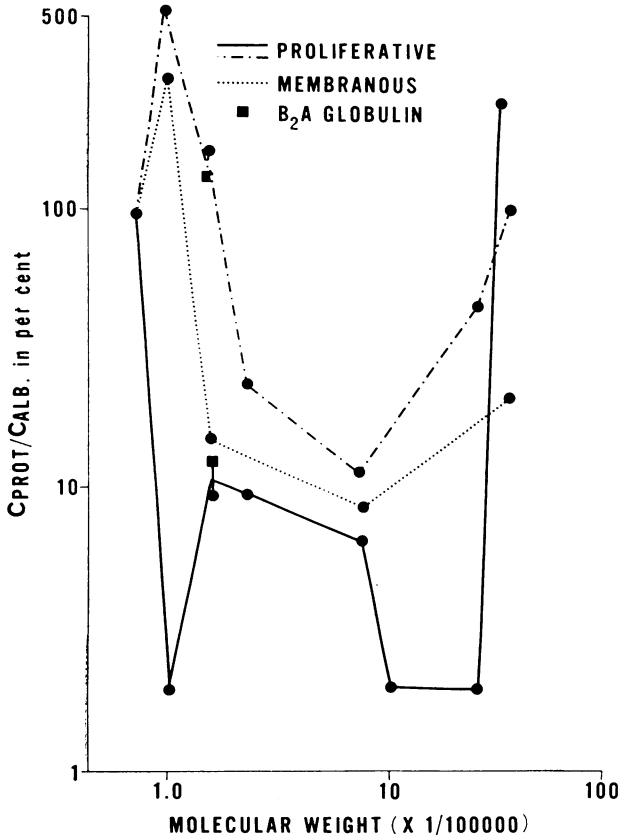

Fig. 3.—Selectivity slopes of urinary proteins from three other patients showing the degree of non-linearity that can be observed in some cases.

depicted in Fig. 3 are observed. Comparative analysis of such polymorphic regression curves is obviously meaningless, since true linearity cannot be established by statistical methods.

Immunoelectrophoresis

In Fig. 4 an immunoelectrophoretic study of normal serum (NHS) is compared with serum and concentrated urine of a patient (DC) with proliferative glomerulonephritis. Many precipitin bands were found in the urine and there was a

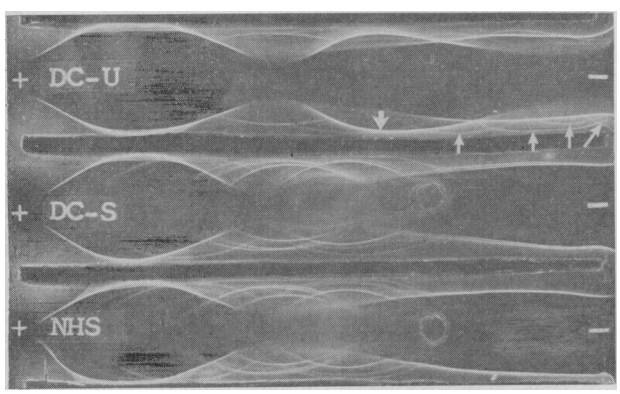

Fig. 4.—Immunoelectrophoretic study of a patient with proliferative glomerulonephritis. DC-U: patient's concentrated urine (× 120); DC-S: patient's serum; and NHS: normal human serum. Immunoelectrophoresis of the urine revealed 13 immune precipitates. Also, in the urine the importance of the transferrin (↓) immune reaction should be noted as well as the presence of four additional bands (↑) in the gamma region.

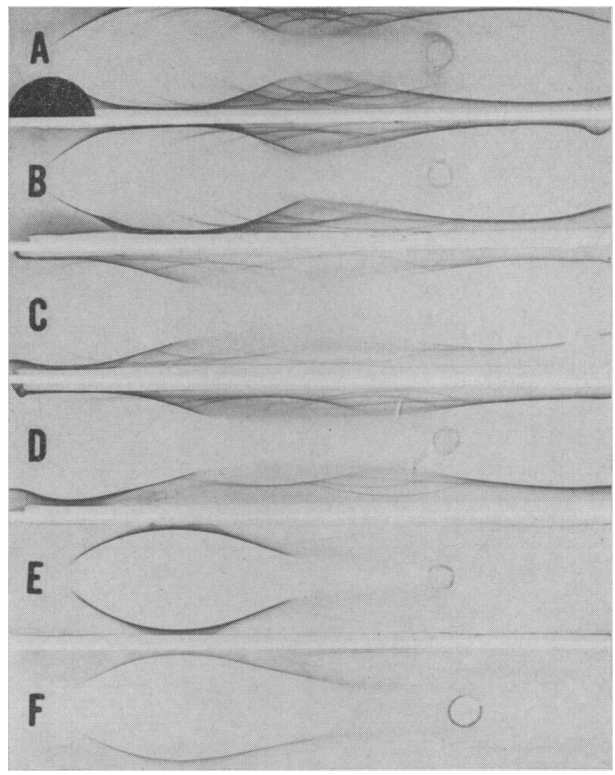

Fig. 5.—Immunoelectrophoretic studies of urinary proteins. A: Normal serum. B, C, D: Concentrated urine specimens from patients with membranous glomerulonephritis. E, F: Concentrated urine specimens from patients with proliferative glomerulonephritis.

particularly strong immune reaction for transferrin. It appears to be almost equivalent to that for albumin, a finding in agreement with the immunodiffusion studies in this patient.

In Fig. 4, four short and distinct precipitates of different mobilities can be seen localized in the cathode region. No cross-reaction is detected either between the precipitates or with the IgG band. Precipitates of similar appearance have been observed frequently but not consistently in other urine specimens. That they are not an artefactual consequence of the concentration procedure is evidenced by their absence from similarly treated serum samples. Bands with similar configuration have been reported in urine by others.[13-15] Their origin has been ascribed either to fragmented molecules of 7S-gamma globulin possibly degraded by an enzyme in urine[14] or to low-molecular-weight gamma globulins normally present in very low concentration in plasma and undetectable by immunoelectrophoresis.[15-17] The recent isolation from plasma of a micromolecular 7S-gamma globulin[18] favours the latter theory.

Fig. 5 illustrates the great variation in the number of proteins present in urines. In some specimens precipitates were almost as numerous as in serum, whereas in others there were only a few fractions. This observation is in agreement with the findings obtained by the immunodiffusion technique (Table II).

In all, 13 urine specimens were analyzed by immunoelectrophoresis (Fig. 6). Albumin, orosomucoid, ceruloplasmin, transferrin, IgA and IgG were the protein fractions most frequently observed in the urines. All have a molecular weight of less than 200,000. By immunoelectrophoresis, as by immunodiffusion, there was a discrepancy between the findings for some proteins with similar molecular weights. For example, IgA and IgG were detected in three and 11 of 13 specimens, respectively. Ceruloplasmin, whose molecular weight (154,000) is slightly less than that of IgG (160,000), was present in only six specimens. Orosomucoid, with the lowest molecular weight (44,000), was observed only eight times.

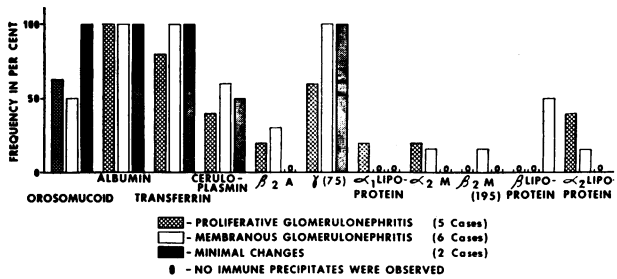

Fig. 6.—Immunoelectrophoretic study of urinary proteins.

On the other hand, proteins with a molecular weight equal to or higher than 200,000 were found infrequently (Fig. 6). IgG (M.W. 160,000) was found in 11 specimens, α_1-lipoprotein (M.W. 200,000) in only one. These data are also in accord with the immunodiffusion studies.

Analysis of the immunoelectrophoretic data in relation to the renal histologic findings did not reveal any significant difference between proliferative and membranous glomerulonephritis (Fig. 6). Comparative studies on SLE patients were also inconclusive. These conclusions are based on inspection of the data only, as they derive from a sample too small to analyze statistically.

Discussion

Until recently, concepts of the glomerular filtration of proteins were based on the work of Lambert, Grégoire and Malmendier.[19-20] From albumin perfusion experiments in the normal dog they concluded that the glomerular capillary wall acted as an ultrafiltration membrane with pores whose diameter approximated 36 A°. Using dextrans of different molecular sizes, Arturson and Wallenius[21] came to a similar conclusion.

When electrophoretic methods became widely available, the investigation of glomerular permeability was extended. For example, Hardwicke and Squire[22] estimated clearances of α and γ globulins in patients with proteinuria. Conclusions derived from electrophoretic studies have now been reexamined as a result of the recent application of immunologic methods capable of demonstrating the high degree of complexity of both serum and urine proteins.

Because immunoelectrophoresis was unsuitable for quantitative purposes, Blainey *et al.*[3] and Hardwicke and Soothill[4] adapted the double diffusion technique in agar gel to the measurement of certain specific plasma and urinary proteins. The clearance of six different proteins was estimated by this method in 38 patients with proteinuria.[4] The results of the protein clearances were analyzed in relation to the histologic changes found in the renal biopsies. In patients with membranous glomerulonephritis they found an increased glomerular permeability to high-molecular-weight proteins. A lesser increase in glomerular permeability was noted in the presence of proliferative changes. In cases with minimal histologic changes, only small-molecular-weight proteins were detected in the urine; these patients were classified as having the most selective proteinuria. From these results it was concluded that in some cases of the nephrotic syndrome the "pores" present in the basement membrane were so altered as to facilitate filtration of large proteins.

In the present study, clearances of seven proteins were measured in 15 patients and the results were expressed as a percentage of $C_{albumin}$. The results appear to differ in many respects from those reported by Hardwicke and Soothill.[4] No consistent difference could be detected in protein clearances from patients with proliferative glomerulonephritis, compared to those with membranous glomerulonephritis. Nor was there a consistent pattern in patients with SLE. Similar results were found in immunoelectrophoretic studies. In neither histologic group could a distinctive pattern of urinary proteins be clearly delineated.

Recently Joachim *et al.*[12] reported a similar study on 48 adult patients with proteinuria. Selectivity of the glomerulus to proteins was estimated according to slopes derived from the linear regression lines of relative protein clearances plotted as ordinates against respective molecular weights (see "Results"). Contrary to the findings of Hardwicke and Soothill,[4] these investigators noted that in general the highest selectivity was found when the glomerular basement membrane was thickened. Less selective proteinurias were observed in patients with chronic nephropathy. In their investigation of 28 nephrotic children, Cameron and White[23] reached similar conclusions.

Such conflicting results from several groups of workers deserve comment. Although we studied few patients, our data cast some doubt on the validity of expressing selectivity of proteinurias in terms of linear regression; moreover, extreme variations of protein clearances were noted even among patients with similar histologic findings (Table II). In many cases the curves departed so greatly from linearity that regression analysis was not attempted (Fig. 3).

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TABLE III.—RECALCULATION OF THE CRUDE DATA FROM TABLE V, IN PAPER BY JOACHIM *et al.*[12]

Patient	*Slope (regression coefficient)* ($-b$)	*Angle* θ	*95 per cent confidence limits of* θ
1 A.....	+2.219000	65° 45′	74° 54′ and 36° 15′
1 B.....	+2.345837	66° 55′	79° 14′ and 0° 0′
2 A.....	+3.021702	71° 41′	80° 15′ and 15° 41′
2 B.....	+2.673874	69° 29′	74° 38′ and 59° 42′
3 A.....	+2.349240	66° 56′	76° 1′ and 34° 27′
3 B.....	+2.620894	69° 7′	70° 30′ and 67° 31′
4 A.....	+1.461658	55° 37′	71° 14′ and 1° 8′
4 B.....	+1.893694	62° 10′	73° 48′ and 19° 3′
5 A.....	+1.517279	56° 37′	72° 56′ and 12° 29′
5 B.....	+1.491555	56° 10′	69° 33′ and 16° 47′

In Table V of their paper Joachim *et al.*[12] published crude data for two replicate determinations on six proteins in each of five studies. A straight line was estimated by the method of least squares and the slope was determined. The angle (θ) was derived from the slope, the tangent of θ, and was used to express the degree of selectivity. In their study they did not indicate the confidence limits of these coefficients. We have used the crude data in their Table V to recalculate the linear regression coefficients (tangents) and their 95% confidence limits (Table III). We have transformed these slopes into their appropriate angles (θ) and 95% confidence limits. From these calculations of the 95% confidence limits it is clear that the claim of these authors that an angle of 13° can be used to differentiate proteinurias of high and low selectivity is not justified. This is not surprising, as the regression lines were estimated from five or at most six individual points, for each of which the coefficient of variation of the estimate is known to be between 14% and 25%.[9] For these reasons we conclude that selectivity slopes cannot be calculated from the U/P data with precision—at least when this method of estimating U/P ratios is used. Thus the separation of cases with low selectivity from cases with high selectivity is highly questionable.

Some of these data call for further comment. That large-molecular-weight (>200,000) proteins were rarely detected even in concentrated urine specimens might be explained, in part at least, by a technical artefact. Elsewhere we have shown that β-lipoprotein, IgM and α_2-macroglobulin are frequently lost or greatly diminished as a result of the techniques used in concentrating the urine specimen.[8] Other authors have found large-molecular-weight proteins uncommonly in urine from patients with the nephrotic syndrome[24, 25] and healthy subjects.[13, 25, 26] A glomerular barrier to the passage of large protein molecules may indeed exist, as has been suggested[3, 4, 12] even when renal damage is present, but in none of the reported studies has the role of technical artefacts been excluded.

It has been suggested that the permeability of the glomerular basement membrane in disease is related directly to the molecular weight of the proteins.[3, 4, 12] Very superficially, our results might seem to be consistent with such a view; but the theory would need to be flexible to explain the appearance of the large-molecular-weight α_2-lipoprotein in the urine of three subjects. In addition, it does not appear to be rigidly applicable to the data on proteins with a molecular weight less than 200,000. The clearance of proteins with molecular weights greater than that of albumin was greater than $C_{albumin}$ in 11 of 90 determinations (Table II). The example of transferrin is instructive in this respect, since the U/P ratio for transferrin (mol. wt.: 90,000) was equal to or higher than that of albumin (mol. wt.: 69,000) in eight of 15 measurements. Although they did not comment on its significance, Joachim *et al.*[12] reported similar data in seven of 10 determinations. The lack of relationship with molecular weight is further illustrated by a comparison of the data for IgG and IgA (Table II)—proteins whose molecular weight is almost identical. In the study reported by Joachim *et al.*[12] the differences were even more striking. The U/P ratios of IgA were considerably less than those of IgG in eight of 10 reported estimations. Thus it appears that molecular weight does not, *per se*, play an exclusive role in regulating glomerular filtration of proteins.

In considering these results two other possible artefacts must be borne in mind: (1) There is a considerable standard error of estimate in the method used.[9] More accurate immunologic techniques are clearly desirable to increase precision. (2) It is now known that the 7Sγ (IgG) globulin molecule in urine may be much smaller than that in serum.[15, 17] Thus even with precise immunologic techniques there must be doubts about the assumption that the molecular weights of the proteins in urine and serum are identical—an assumption inherent in all these studies.

The results of the present studies cannot be explained satisfactorily on the basis of glomerular filtration alone, and some consideration must be given to the possible role of tubular reabsorption of proteins. Much evidence indicates that proteins are reabsorbed by the tubules,[27-33] and recent studies strongly suggest that after reabsorption both foreign and homologous proteins are broken down by proximal tubular lysosomes.[32, 33] Reabsorption of albumin has been demonstrated in the proximal tubules of Necturus.[34] Reabsorption of protein by the proximal tubules has also been shown, using a micropuncture technique, by Carone and von Haam[35] in both normal rats and rats made nephrotic with aminonucleoside. These authors found that the protein content of normal glomerular, proximal and distal tubular samples was, on the average, 73, 10.5 and 35 mg./100 ml., respectively. In nephrotic rats similar results were

found in glomerular and proximal tubular samples; only in the distal tubular samples was the protein concentration significantly increased. They concluded that in the normal rat a small but significant amount of protein was filtered through the glomerulus and was largely reabsorbed by the renal tubules. The increased protein loss seen in proteinuric animals was thought to be the consequence of decreased tubular reabsorption of protein principally by distal and collecting tubules, and not a result of enhanced glomerular filtration.

Glomerular filtration is a complex phenomenon which is dependent, *inter alia,* on the physicochemical laws of both filtration and diffusion. The discrepancy between the data for IgG and IgA might, for example, be explained on the basis of the distinct physicochemical properties of these two proteins. On the other hand it might be necessary to postulate that the transferrin molecule was modified in some way so as to make it more readily filterable than albumin; and there is no evidence for such a hypothesis. It seems more likely that the tubular cells play a significant role in determining the concentration and composition of the urinary proteins.

It is probable that the physicochemical laws regulating glomerular filtration of proteins do not apply to the reabsorptive mechanism of tubular cells where micropinocytosis and protein transport through cellular membranes may be factors of major importance. Thus the molecular size of proteins is likely to have less influence on the segregating activities of tubular reabsorption compared with those of glomerular filtration. Such a hypothesis would explain the inconsistent results of protein clearances observed in the present study.

SUMMARY

Using the immunodiffusion technique, the clearances of seven different proteins were measured in 15 patients with proteinuria. SLE was present in six of these patients. Immunoelectrophoretic studies of the concentrated urines were performed in each case. The renal histology was evaluated by means of a biopsy.

There was no correlation between the histologic changes and the urinary excretion of proteins. This observation was confirmed by both immunodiffusion and immunoelectrophoretic techniques. No specific patterns of urinary proteins were found in patients with SLE.

High-molecular-weight proteins were seldom detected in urine even when the glomerular basement membrane was definitely thickened. Low-molecular-weight proteins were frequently observed, but their clearances were extremely variable. These results cast doubt on the validity of the suggestion of some authors that protein clearances can be used for diagnosis or prognosis in renal diseases.

The data from the present study do not support the view that glomerular filtration is the sole factor responsible for the final pattern of urinary proteins. Tubular reabsorption of proteins is likely to be another important factor.

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THE HAPPY ADJUSTMENT

There is a phase of practice from which none can hope to escape. Every practitioner knows that he sustains toward his patients a relationship other than that implied in the administration of drugs, in the performance of operations or the correct adjustment of a mechanical appliance. There is a personal relationship which he can not avoid, one which implies faith, confidence and affinity or the opposites. His success in his work must depend largely upon the happy adjustment of these relations.—B. E. McKenzie, *Canad. Med. Ass. J.*, 6: 118, 1916.